Project:

Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Turbidimetric LAL Assay

Subtitle

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1 Introduction

This document describes a protocol for a quantitative detection of Gram negative bacterial endotoxin in nanoparticle preparations using a kinetic turbidimetric Limulus Amebocyte Lysate (LAL) assay.

2 Principle of the Method

This method relies on an *in vitro* endotoxin test which utilizes a *Limulus Amebocyte* Lysate (LAL), an extract of blood cells (amebocytes) from the horseshoe crab. The method is designed to detect endotoxin activity photometrically with an automated tube reader incubating the reaction mixture at controlled temperature of 37°C.

Gram-negative bacterial endotoxin catalyzes the activation of proenzyme in the Limulus Amebocyte Lysate. Bang[1] observed in 1956 that the infection of the horseshoe crab *Limulus polyphemus* with Gram-negative bacteria resulted in intravascular coagulation, as a result of a reaction between endotoxin and a clotting protein in amebocytes of *Limulus*[2]. This method is based on the activation of the LAL proenzyme in the presence of endotoxin. As a result of the following cascade of enzyme activation steps, turbidity of the reaction mixture increases due to the initiated coagulation process. The development of turbidity is measured using an automated tube reader. The time to reach a specific increment in turbidity (referred to as "onset time") is determined. Higher endotoxin concentrations give shorter onset times. Concentration of endotoxin in a test sample is calculated from a standard curve prepared with series of known concentrations of a certified endotoxin standard. The method as performed here relies on Limulus Amebocyte Lysate PYROTELL®–T by Pyroquant Diagnostik GmbH, a subsidiary of Associates of Cape Cod, Inc. (ACC) [3]. Data analysis is performed using PYROS® Software (ACC).

3 Applicability and Limitations (Scope)

This SOP was developed to determine and quantify endotoxin contamination of different nanomaterials. This SOP was created according to ISO/EN 297014 adapted for the analysis nanomaterials[4] as well in consideration of references 5 - 7. Procedures were described according to the supplier's instruction for the use of the Kinetic Turbidimetric LAL Assay Pyrotell®-T [3]. Use Pyrotell®-T for *in vitro* diagnostic purposes only. Do not use it for the detection of endotoxemia [3].

4 Related Documents

Table 1:

Document ID	Document Title
NCL Method STE-1.2	Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Turbidity LAL Assay
EUNCL-STE-001.2.2	Detection and Quantification of Gram Negative Bacterial Endotoxin Contamination in Nanoparticle Formulations by Kinetic Turbidimetric Microplate LAL Assay

5 Equipment, Materials and Reagents

5.1 Equipment

5.1.1 Microcentrifuge

5.1.2 Refrigerator, 2-8°C

5.1.3 Freezer, -20°C

5.1.4 Vortex mixer

5.1.5 automated PYROS Kinetix® Flex Tube Reader 660 nm, temperature controlled 37°C, (ACC)

5.2 Materials

5.2.1 Pyrogen-free microcentrifuge tubes, 1.5 mL (e.g. Eppendorf BioPure®)

5.2.2 Pyrogen-free pipettes and barrier tips covering the range from 0.01 to 1 mL (e.g. Eppendorf; Sarstedt Biosphere®)

5.2.3 Pyrogen-free dispenser tips, 50 µL increment (e.g. Eppendorf BioPure®)

5.2.4 Repeating pipettor (e.g. Eppendorf)

5.2.5 Disposable endotoxin-free glass (borosilicate) reaction tubes for Tube Reader 7,9 x 75 mm (ACC, TK100) or equivalent

5.2.6 Disposable endotoxin-free glass (borosilicate) dilution tubes 13 × 100 mm (Lonza N207) or 12 x 75 mm (ACC TB240) or equivalent

5.2.7 Parafilm® “M” Laboratory film (Pechiney Plastic Packaging)

5.3 Reagents

5.3.1 Test nanomaterial

5.3.2 LIMULUS AMEBOCYTE LYSATE PYROTELL®–T For The Detection And Quantification Of Gram Negative Bacterial Endotoxins (PYROQUANT DIAGNOSTIK, Associates of Cape Cod, Inc. (ACC))

5.3.3 Control Standard Endotoxin (CSE) (PYROQUANT DIAGNOSTIK GmbH, ACC)

5.3.4 Glucashield® (1→3)-ß-D-Glucan Inhibiting Buffer (PYROQUANT DIAGNOSTIK GmbH, ACC)

5.3.5 LAL Reagent Water (PYROQUANT DIAGNOSTIK GmbH, ACC)

5.3.6 Sodium hydroxide, 0.1 N, endotoxin free (e.g. Acila 1712200)

5.3.7 Hydrochloric acid 0.1 N, endotoxin free (e.g. Acila 1712300)

5.3.8 Endotoxin free water (e.g. ACC W0051; Acila 1715050; or equivalent)

5.4 Reagent Preparation

Store all provided reagents of the kit at 2-8°C. Prior to use allow reagents to equilibrate to room temperature[3].

5.4.1 Preparation of the LAL Reagent

The assay reagent is provided as lyophilized mixture of LAL. Reconstitution should be performed according to the supplier' s recommendation, in LAL grade water (LAL Reagent Water) or Glucashield® Buffer, both available from the manufacturer as separate components. EUNCL preferred method is the application of Glucashield® buffer which allows to exclude interference from β-1,3-Glucans which is very common in nanomaterials produced using filtration steps. Substances containing β-1,3-Glucans are important sources of false-positives, a synergistic response (i.e. enhancement) is frequently seen with β-Glucan containing samples spiked with endotoxin. The usage of a Glucashield® buffer is therefore indicated whenever β-1,3-Glucan contamination is expected [3].

Reconstitute Pyrotell®-T LAL Reagent only immediately before use. Add the volume as indicated on the vial label (usually is 5 mL). In case more than one vial for a large number of samples is required, pool reconstituted reagent of two or several vials before use. Exercise extreme caution to avoid formation of air bubbles. Do not vortex the reconstituted lysate. Pipette with caution. Mix only by very gently swivel to avoid foaming. In case of bubbles allow to clear before use. Cover the vial with Parafilm M® when not in use.

Store the lyophilized Pyrotell®-T LAL Reagent at -20 to 8°C until expiration date on the label. Reconstituted LAL Reagent should be used promptly and is stable for up to 24 hours at 2 to 8°C, or can be stored at or below -20°C for up to three months if frozen immediately after reconstitution. Freeze and thaw the reconstituted LAL Reagent only once (ACC) [3].

5.4.2 Endotoxin Control Standard Endotoxin stock solution

E.coli lipopolysaccharide (LPS) supplied by ACC is a certified control standard endotoxin (CSE) provided as a lyophilized powder. Store at 2-8°C before reconstitution. Prepare a stock solution of approximately 1000 EU/mL by reconstitution of the Control-Standard Endotoxin (CSE, purified Endotoxin extract of *E. coli* O113:H10 [3]) in LAL Reagent water.

Remove the metal seal from the vial, break the vacuum by lifting the stopper just enough to allow air to enter, and aseptically remove the stopper. Add approximately 3.5 - 5.0 mL LAL Reagent water with caution to the CSE vial. The final volume and concentration for a given CSE vial has to be calculated from the information provided by the Certificate of analysis. The certified potency of the specific lot of CSE in use with a specific lot LAL reagent is indicated relative to the current FDA or USP reference standard endotoxin (Certificate available from the supplier ACC[3]).

During reconstitution and prior to use, the stock solution should be vortexed vigorously for 30-60 sec, with 5-10 min settling times, over a 30-60 min time frame, and allowed to equilibrate to room temperature. Vortex the CSE for at least 30 seconds each time immediately before taking an aliquot for usage to make appropriate dilutions.

The reconstituted stock CSE solution is stable for 4 weeks stored at 2-8°C, do not freeze CSE [3]. Before usage of the stored stock bring to room temperature and mix vigorously for 15 minutes, in order to release endotoxin that tends to attach to the glass surface.

5.4.3 Preparation of Endotoxin calibration standards

For the Pyrotell®-T LAL Endotoxin the lowest detection limit λ is 0.001 EU/mL with a detection range up to 1 EU/mL[3], when readout is performed with the tube reader. Adjustment of the assay detection range can be defined by limiting the range of endotoxin concentration used to construct the standard curve.

Label disposable pyrogen-free glass dilution tubes for the endotoxin dilutions. Prepare a serious of dilutions starting with stock the CSE concentration. Each dilution should be vigorously vortexed for at least 1 minute before proceeding with the next step of the dilution series. The following scheme is an example how to prepare the endotoxin calibration standards, alternative dilution schemes and range may be used.

Dilution scheme for preparation of a series of endotoxin standard dilutions

Sample	Nominal Concentration (EU/mL)	Preparation Procedure	
Int. A	100*	100 µL Stock CSE	+ 900 µL LAL reagent water
Int. B	10	100 µL of Int. A	+ 900 µL LAL reagent water
Cal. 1	1.0	100 µL of Int. B	+ 900 µL LAL reagent water
Cal. 2	0.1	100 µL Cal. 1	+ 900 µL LAL reagent water
Cal. 3	0.01	100 µL Cal. 2	+ 900 µL LAL reagent water
Cal. 4	0.001	100 µL Cal. 3	+ 900 µL LAL reagent water

* This is provided only as an example; numbers shown in the table above are calculated based on a stock CSE concentration of 1000 EU/mL.

Each sample should be vigorously vortexed for at least one minute prior to use.

5.5 Assay Control Reactions

5.5.1 Preparation of Inhibition/Enhancement test using a Positive Product Control (PPC)

To determine interferences with the product each test sample concentration is prepared with a defined amount of standard endotoxin CSE. The nominal endotoxin concentration spiked into the PPC should equal that of a standard dilution from the middle of the standard curve. The following schemes are examples how to prepare the endotoxin control reactions, alternative dilution schemes and concentrations may be used.

Dilution scheme example: Preparation of Positive Product Controls (PPC)

Sample	Nominal Concentration (EU/mL)	Preparation Procedure	
Int. A **	100 *	100 μL Stock CSE	+ 900 μL LAL reagent water
Int. B **	10.0	100 μL of Int. C	+ 900 μL LAL reagent water
Int. C **	1.0	100 μL of Int. D	+ 900 μL LAL reagent water
PPC	0.05	25 μL of Int. C	+ 475 μL nanoparticle suspension***

* This is provided only as an example; numbers shown in the table above are calculated based on a Stock concentration of 1000 EU/mL.

** Intermediate solutions A, B, C are prepared to make control dilutions and are not used in assay.

*** The concentration of nanoparticles should be equal to one assayed in a test-sample.

You will need to prepare IEC for each nanomaterial dilution assayed in this test.

5.5.2 Quality Controls

For the quality control, the same final concentration of standard endotoxin is diluted in to LAL reagent water.

Dilution scheme example: Preparation of Quality Controls

Sample	Nominal Concentration (EU/mL)	Preparation Procedure	
Int. A **	100 *	100 μL Stock CSE	+ 900 μL LAL reagent water
Int. B **	10.0	100 μL of Int. C	+ 900 μL LAL reagent water
Int. C **	1.0	100 μL of Int. D	+ 900 μL LAL reagent water
QC	0.05	50 μL of Int. C	+ 950 μL LAL reagent water

* Numbers shown in the table above are calculated based on a Stock concentration of 1000 EU/mL.

** Intermediate solutions A, B are prepared only to make control dilutions and are not used in assay.

5.5.3 Negative Control

Use endotoxin free LAL reagent water or respective diluent buffer of the samples as a negative control.

Transfer 200 μL of each prepared Assay Control Reaction into fresh endotoxin test glass tubes in duplicate.

6 Procedure

6.1 General remarks

Most importantly microbial or endotoxin contamination of all samples and materials having contact with the sample and all used test reagents must be avoided by careful handling and technique.

6.2 Preparation of Study Samples

Study samples should be reconstituted in either LAL reagent water or sterile, pyrogen-free PBS. The pH of the study sample should be checked. It may be necessary to adjust the pH of the sample to within the range 6.0–8.0 using either sterile endotoxin-free sodium hydroxide or hydrochloric acid. Do not adjust the pH of unbuffered solutions. Pyrogen-free Tris buffer may also be used to prepare samples for endotoxin detection in place of water as a sample diluent to adjust pH of highly acidic or basic samples. To avoid sample contamination always remove a small separate aliquot from the prepared sample to measure pH. If the sample was prepared in PBS or other diluent, the blank diluent should be tested for endotoxin contamination as control. The concentration of nanomaterial is unique to each formulation. The goal of this test is to measure endotoxin level per mg of the test formulation, which commonly refers to the active pharmaceutical ingredient (API), but may also be measured in mg of total formulation or total element (e.g. gold or silver). The sample should be tested from the stock using several dilutions not exceeding the Maximum Valid Dilution (MVD).

To determine the MVD three parameters are needed: endotoxin limit (EL), the sample concentration and assay sensitivity (λ). EL is calculated according to the formula:

$$EL=K/M,$$

where K is maximum endotoxin level allowed per dose (5 EU/kg for all routes of administration except for the intrathecal route, for which K is 0.2 EU/kg) and M is the maximum dose to be administered per kg of body weight per single hour [5]. Note, estimation of EL for nanomaterials used as radiopharmaceutical or as medical device will be different [5]. When the dose information for the test nanomaterial is available based on an animal model (e.g. in mouse), to can be converted into human equivalent dose (HED). To do so the animal dose is divided by the conversion factor specific to each animal species, e.g. 12.3 for mouse. Please refer to guidelines for other conversion ratios [6]. Dose for cancer therapeutics is often provided in mg/m^2 instead of mg/kg. To convert an animal or human dose from mg/m^2 to mg/kg, the dose in mg/kg is divided by the conversion factor of 37, indicated as km (for mass constant). The km factor has units of kg/m^2; it is equal to the body weight in kg divided by the surface area in m^2. Example 74 mg/m^2 /37 = 2mg/kg [6].

The MVD is determined according to the following formula:

$$MVD= (EL \times \text{sample concentration})/ \lambda).$$

For example, when nanoparticle sample concentration is 10 mg/mL and its maximum dose in mouse is 123 mg/kg, the HED is 123 / 12.3 = 10 mg/kg; EL for all routes except intrathecal would be 0.5 EU/mg (5 EU/kg / 10 mg/kg) and MVD would be 5000 ((0.5 EU/mg x 10 mg/mL) / 0.001 EU/mL). In this case, the nanomaterial will be tested directly from stock or at several dilutions not exceeding the MVD of 5000, e.g. 5, 50, 500 and 5000. When the information about the dose is unknown, the highest final concentration of the test nanomaterial is 1 mg/mL, and the MVD is 500.

It is very important to notice that if the dose, route of administration and/or the sample concentration for the test nanomaterial change, the EL and MVD will also change.

Turbid nanoformulations in high concentration may interfere with this assay and therefore should be tested using other versions of LAL (e.g. gel-clot and chromogenic). Turbid formulations with low MVD

will likely interfere with both turbidity and chromogenic LALs and should be tested using the gel-clot and other methods as discussed in reference 7.

6.3 Flow chart

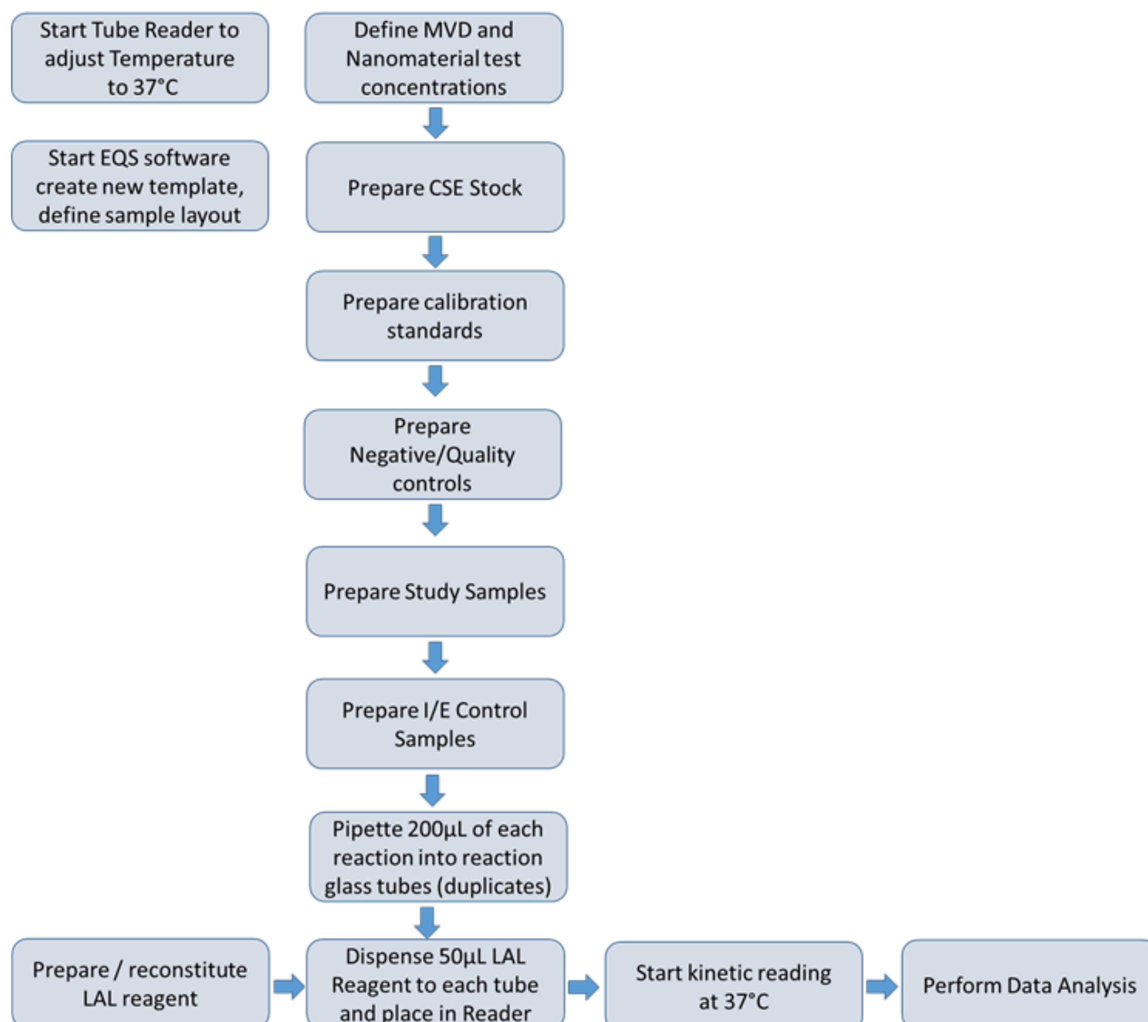

Figure 1: Brief outline of the workflow.

6.4 Measurement Procedure

6.4.1 Turn on the reader instrument at least 30-60 minutes before starting the assay to allow the instrument to warm up to a constant temperature of 37 °C. Set up detection wavelength to 660 nm, which is the mode appropriate for the turbidity LAL.

6.4.2 Start the PYROS® Software and create a new experiment template. Make sure instrument-computer communication is not interrupted. Make sure negative control sample is entered through the negative control panel, not through the sample-panel, otherwise software will not be able to generate report.

6.4.3 Shortly before usage reconstitute the necessary LAL Reagent vial with LAL Reagent Water or Glucashield® buffer (according to the manufacturers instruction), mix only gently (**do not vortex!**) as described above (for details read under -Reagent Preparation-).

6.4.4 Add 200 mL of negative control (water), calibration standards, quality control, IEC and test nanoparticles into pre-labeled glass tubes. Prepare each sample at least in duplicate tubes.

6.4.5 Using a repeating pipette, add 50 mL of LAL reagent to first test vial, vortex it briefly, and insert into test slot in the instrument carousel. Repeat this procedure for other samples, processing one sample at a time. Allow instrument to run each point for no less than 7200 sec to allow time for samples with low amounts of endotoxin to develop. If no detectable endotoxin is present in the sample, the software will mark this sample as "not detected by 7200s".

6.4.6 Note: some lots of the lysate are less sensitive than others, if the sensitivity of a particular lot is low, the time may need to be adjusted to 9600 sec or longer in order to allow the lowest calibrator to develop.

6.4.7 Note: During the measurement time, do not disturb or move the tube reader plate. The laboratory bench supporting the optical reader should be free from excessive vibration (e.g. no vortex, centrifuges or similar working equipment).

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 Assay Acceptance Criteria

7.1.1 Linear regression algorithm is used to construct the standard curve. Precision (%CV) and accuracy (PDFT) of each calibration standard and quality control should be within 25%.

7.1.2 At least three calibration standards should be available for assay to be considered acceptable.

7.1.3 The correlation coefficient of the standard curve must be at least 0.980.

7.1.4 If quality controls fail to meet acceptance criterion described in 7.1, run should be repeated.

7.1.5 If standard curve fails to meet acceptance criterion described in 7.1 – 7.3, the run should be repeated.

7.1.6 Precision of the study sample should be within 25%.

7.1.7 Precision of inhibition/enhancement control should be within 25%.

7.1.8 Spike recovery indicative of the accuracy of the inhibition/enhancement control should between 50 and 200% [5]. Spike recovery less than 50% is indicative of inhibition; that above 200% is indicative of either endotoxin contamination or enhancement.

7.1.9 If sample interference is detected, the assay results for this sample are invalid. Other tests should be considered as discussed in reference 7.

7.2 Sample Acceptance Criteria

7.2.1 Endotoxin level in the sample is acceptable if it is within the EL calculated for the given formulation and application (please refer to section 6.2 and reference 7 for details).

8 Health and Safety Warnings, Cautions and Waste Treatment

Use Pyrotell-T for in vitro diagnostic purposes only. Do not use it for the detection of endotoxemia. The toxicity of the reagent has not been determined; thus, caution should be exercised when handling Pyrotell-T ([4])

Inform yourself about the content and sample material and all relevant safety issues concerning the samples before unpacking and handling of any received sample.

Always wear adequate personal protective equipment, in particular protective laboratory coat, gloves and safety glasses and take all necessary precautions to protect yourself and others. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with the products. Use respiratory protection whenever advisable. Open the sample vials only under sterile conditions of the sterile work bench in order to avoid sample spilling and contamination. Take all necessary precautions to avoid any further sample spilling in case of damaged sample container. Waste disposal has to be proceeded in a proper form using means adequate for the material specifications, in compliance with the laboratory regulations and general regulatory conditions according to applicable legal regulations.

9 Abbreviations

API active pharmaceutical ingredient

BW blank water

CSE control standard endotoxin

CV coefficient of variation

EL Endotoxin Limit

EU endotoxin unit

FDA Food and Drug Administration

HED human equivalent dose

HCl hydrochloric acid

IEC inhibition/enhancement control

LAL Limulus Amebocyte Lysate

LPS lipopolysaccharide

MVD Maximum Valid Dilution

NaOH sodium hydroxide

PBS phosphate buffered saline

PDFT percent difference from theoretical

PES polyethersulfone

PPC positive product control

RT room temperature

Tris tris(hydroxymethyl)aminomethane

USP United State Pharmacopeia

10 References

[1] Bang, F.B. A bacterila disease of *Limulus polyphemus*. Bull. Johns Hopkins Hosp. 98:325 (1956)

[2] Levin, J., Bang. F.B. Clottable protein in *Limulus*: its localisation and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19:186 (1968)

[3] PYROQUANT DIAGNOSTIK GmbH; ACC

ACC Pyrotell®-T Turbidimetric Formulation Package Insert (PDF)| Product Info Sheet (PDF); (http://www.acciusa.com)

ACC Control Standard Endotoxin (CSE) Product Inserts downloads: CSE, 0.5µg/vial | CSE, 125µg/vial (PDF); Product Info Sheet (PDF); (http://www.acciusa.com)

ACC Glucashield® (1→3)-ß-D-Glucan Inhibiting Buffer - Reconstitution Products Package Insert (PDF) | Product Info Sheet (PDF); (http://www.acciusa.com)

[4] CEN (Europäisches Komitee für Normung), Nanotechnologien – Endotoxinprüfung an Proben aus Nanomaterial für In-vitro-Systeme – Limulus-Amoebozyten-Lysat-Prüfung (LAL-Prüfung), DIN EN ISO 29701, Ausgabe: 2011-01

[5] USP 34-NF29. <85>. Bacterial Endotoxins. Rockville, MD: United States Pharmacopeia, 2011,Volume 1, 78-81.

[6] FDA Guidance for Industry and Reviewers Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers. December 2002.

[7] US FDA. Guidance for Industry. Pyrogen and Endotoxins testing: Questions and answers, 2012.